

*DISULFIDE INTERCHANGE AND THE THREE-DIMENSIONAL
STRUCTURE OF PROTEINS*

BY DAVID GIVOL,* FRANCESCO DE LORENZO,† ROBERT F. GOLDBERGER,
AND CHRISTIAN B. ANFINSEN

LABORATORY OF CHEMICAL BIOLOGY, NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES,
NATIONAL INSTITUTES OF HEALTH

Communicated January 28, 1965

The crystallographic studies of Kendrew and Perutz and their colleagues have demonstrated that the myoglobins and hemoglobins of several species possess unique tertiary structures.¹⁻² Such structural homogeneity probably characterizes most or all proteins, as indicated by the large body of physical and chemical information now available on many purified preparations. It has been suggested that the three-dimensional conformations of proteins are completely defined by the information present in the linear sequences of amino acids that make up the corresponding polypeptide chains.³ This suggestion has been supported by a number of studies on the reversible denaturation of proteins (summarized in ref. 4). It has been shown that the renatured molecules, both those devoid of covalent cross linkages⁵⁻⁷ and those possessing disulfide bonds which have been reductively cleaved prior to renaturation,⁸⁻¹² exhibit physical and biological properties indistinguishable from those of the native molecules. These studies indicate that the tertiary structure and specific disulfide bonds of a native protein molecule represent the most stable conformation of its polypeptide chain under physiologic conditions.

If the information required for the pairing of half-cystine residues in a polypeptide chain is inherent in the amino acid sequence, one may ask whether interrup-

tion of the chain by cleavage of one or more peptide bonds might seriously modify this information. This could be the case, for example, for a protein that is synthesized as one polypeptide chain (e.g., chymotrypsinogen) and functional as a multichained protein (e.g., chymotrypsin).

We have recently shown that a microsomal enzyme which accelerates the reactivation of the reduced form of bovine pancreatic ribonuclease (RNase) and of egg white lysozyme¹³ is involved in the catalysis of sulfhydryl-disulfide interchange.¹⁴ Thus, the inactive product obtained by oxidation of reduced RNase in urea, containing random sets of half-cystine pairs, is rapidly converted to active RNase by the enzyme. If the "correct" disulfide bonds of a protein are a corollary of native tertiary structure, an enzyme that would catalyze their rearrangement could be used to test the stability of the sequence-directed pairing of half-cystine residues. We present, in this communication, the results of studies on the effect of the disulfide interchange enzyme on several RNase derivatives, chymotrypsin, and insulin. The results are consistent with the idea that the specific disulfide bonds of these proteins were formed according to the information present in single-chained precursors which were subsequently converted, by peptide bond cleavage, to the metastable multichained proteins.

Materials and Methods.—Bovine pancreatic ribonuclease (Sigma Chemical Co.) was reduced as described previously¹³ and was allowed to oxidize either spontaneously in 8 *M* urea at pH 8.2 for 100 hr,¹⁵ or by incubation for 10 min with 10^{-3} *M* dehydroascorbic acid (DHA) (Nutritional Biochemical Corp.) in 0.05 *M* bicarbonate buffer, pH 7.4.¹⁴ After removal of the urea or the DHA under acidic conditions,¹⁴ both preparations were found to contain no free sulfhydryl groups and to be enzymically inactive.

The "C-protein" derivative of RNase was prepared by cleavage of methionyl bonds with cyanogen bromide and subsequent removal of the NH₂-terminal tridecapeptide by gel filtration through a column of Sephadex G-25 (Pharmacia).¹² The extents and rates of reactivation of reduced RNase, and of the oxidized RNase preparations described above, were determined under various conditions by assay of RNase activity at pH 5.0.¹⁶

Chymotrypsinogen A and α -chymotrypsin (3 \times crystallized) were purchased from the Worthington Biochemical Corp. Chymotrypsinogen was activated by incubation with trypsin (see *Results*). The esteratic activity of α -chymotrypsin was determined by the hydrolysis of benzoyl-tyrosyl-ethyl ester (Determatube BTEE, Worthington Biochemical Corp.).¹⁹ The increase in absorbance at 256 m μ was followed during the first 4 min of the reaction. Trypsin (2 \times crystallized, Worthington Biochemical Corp.) was treated with diisopropyl-fluorophosphate to destroy residual chymotryptic activity.²⁰ Beef insulin (low zinc; lot #4096-S36997) and the oxidized B chain and A chain of insulin were a gift from Eli Lilly Laboratories. Beef insulin labeled with I¹³¹ (Abbott Laboratories) was used in a solution of 10 μ g/ml in 0.025 *M* phosphate buffer, pH 8, containing 50 mg of bovine serum albumin (Armour Co.) per ml. Pepsin (2 \times crystallized) was obtained from Worthington Biochemical Corp.

The disulfide interchange enzyme was prepared from beef liver microsomes as described previously, except that Sephadex G-200 filtration was introduced into the procedure following the CM-Sephadex step.¹⁴ The enzyme was assayed routinely by its effect on the rate of reactivation of reduced RNase.¹³

Sephadex G-25 and G-200, DEAE-Sephadex, and CM-Sephadex were purchased from Pharmacia. Urea (Baker Chem. Co.) was recrystallized from 95% ethanol before use. β -Mercaptoethanol (Eastman Co.) was used without further purification.

Immunoassay of I¹³¹-labeled pork insulin was performed essentially as described by Berson *et al.*,²¹ except that ascending chromatography in 0.025 *M* phosphate buffer, pH 8, was used instead of electrophoresis. The samples (0.5 ml containing 0.5 μ g of I¹³¹-labeled insulin) were incubated with 0.05 ml of guinea-pig antiovine insulin antiserum (diluted 1:40 with 0.025 *M* phosphate buffer, pH 8.0, containing 50 mg bovine serum albumin per ml) for 48 hr at 4° and 0.2

ml was chromatographed on Whatman 3 MM paper for $3\frac{1}{2}$ hr at 4° . Radioactivity was measured with a 4-Pi automatic windowless paper chromatogram scanner (Atomic Accessories, Inc.).

Changes in the pairing of half-cystine residues in insulin were examined qualitatively on peptide maps after peptic digestion. The protein was dissolved in 5% formic acid at a concentration of 10 mg/ml and pepsin (0.1 mg in 10 μ l 5% formic acid) was added. After 6 hr of incubation at 37° a second aliquot of pepsin was added. The reaction was stopped, after a total incubation time of 16 hr, by freezing and lyophilization. The lyophilized material was dissolved in water at a concentration of 50 mg/ml, and 0.05 ml were applied to Whatman 3 MM paper. Descending chromatography was performed in the organic phase of butanol:acetic acid:water (4:1:5), and electrophoresis was carried out at 2500 v in pyridine acetate buffer, pH 3.6, for 2 hr. The peptide maps were prepared in duplicate. One was stained with ninhydrin and the other with cyanide-nitroprusside for the detection of peptides containing disulfide bonds.²² Oxidation with performic acid was carried out according to the method of Hirs.²³

A Beckman/Spinco model 120 amino acid analyzer was utilized for amino acid analyses. Samples for analysis were hydrolyzed in constant boiling HCl in evacuated, sealed tubes for 22 hr at 110° . Protein concentrations were determined by the method of Lowry *et al.*²⁴ Free sulfhydryl groups were determined with 5,5'-dithiobis(2-nitrobenzoic acid) (Aldrich Chemical Co.).²⁵

Results.—Ribonuclease derivatives: The enzymically inactive products obtained by the oxidation in 8 *M* urea or with DHA have been shown to be rapidly activated by the disulfide interchange enzyme in the presence of β -mercaptoethanol (optimal concentration, 10^{-3} *M*) (Fig. 1, curves 1 and 2), as contrasted with the long periods (16–24 hr) required without the enzyme.¹⁶ The requirement for β -mercaptoethanol in the reactivation mixture could be abolished by previous partial reduction of these molecules (Fig. 1, curve 3).¹⁴ Since we have shown that the enzyme does not accelerate the oxidation of sulfhydryl groups,¹⁴ and since it activates “incorrectly” cross-linked RNase as well as fully reduced RNase, it may be concluded that the process catalyzed is a sulfhydryl-disulfide interchange.

The effect of the enzyme on the “C-protein” derivative of RNase described by Gross and Witkop¹⁷ is illustrated in Figure 2. The C-protein is composed of three polypeptide chains held together by one intrachain and three interchain disulfide bonds, all present in the original RNase molecule. The aggregation and precipitation produced by the enzyme in the presence of 10^{-3} *M* β -mercaptoethanol was so rapid that the β -mercaptoethanol concentration had to be diminished to 10^{-4} *M*. At the latter level the concentration of β -mercaptoethanol was less than 5 per cent that of half-cystine residues in the C-protein employed (4 mg protein/ml). Thus, the precipitation cannot be due to simple reduction. No precipitation occurred over a 24-hr period in the presence of either enzyme alone or β -mercaptoethanol alone (10^{-3} or 10^{-4} *M*) under these conditions (0.1 *M* Tris, pH 7.2). (Precipitation did occur after 1 hr at pH 7.8 in the presence of 10^{-3} *M* β -mercaptoethanol alone.) The enzyme-catalyzed aggregation and precipitation of C-protein is presumably caused by disulfide interchange, which leads to random pairing of half-cystine residues forming a cross-linked network of chains.

In previous experiments on the reactivation of reduced RNase it was difficult to demonstrate enzyme activity at low ratios of enzyme to reduced RNase¹³ since concentrations of the substrate (reduced RNase) greater than 0.02 mg/ml led to extensive intermolecular disulfide bonding.²⁶ In the experiments with C-protein and with insulin, described below, catalysis by the disulfide interchange enzyme is easily demonstrated at weight ratios of enzyme to substrate of less than 1:100.

Chymotrypsin: Chymotrypsin rapidly inactivates the disulfide interchange en-

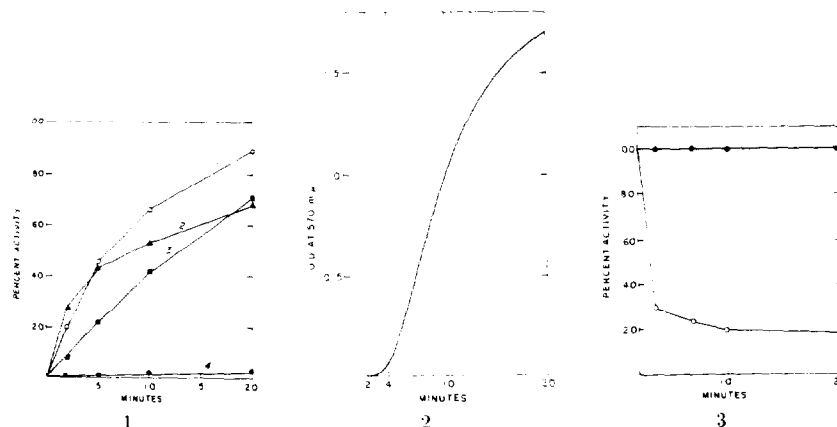


FIG. 1.—Reactivation of oxidized RNase derivatives. Curve 1: DHA-oxidized RNase, $10^{-3} M$ β -mercaptoethanol. Curve 2: Urea-oxidized RNase, $10^{-3} M$ β -mercaptoethanol. Curve 3: Urea-oxidized RNase containing 2-4 sulfhydryl groups per mole.¹⁴ Curve 4: DHA-oxidized or urea-oxidized RNase with either $10^{-3} M$ β -mercaptoethanol or the disulfide interchange enzyme. All incubations were carried out at 37° in 0.1 M Tris, pH 7.5, and contained $20 \mu\text{g}$ of RNase derivative per ml and (except as indicated for curve 4) $20 \mu\text{g}$ of the disulfide interchange enzyme per ml.

FIG. 2.—Treatment of "C-protein" with the disulfide interchange enzyme. The incubation mixture contained 4 mg of "C-protein" and $80 \mu\text{g}$ of the disulfide interchange enzyme in 1 ml of 0.1 M Tris, pH 7.2, $10^{-3} M$ β -mercaptoethanol. Turbidity was followed with a Cary model 15 spectrophotometer.

FIG. 3.—Inactivation of chymotrypsin by the disulfide interchange enzyme. The upper curve (●—●) was obtained with an incubation mixture containing $10 \mu\text{g}$ of chymotrypsin, diluted from a urea solution (see text), in 1 ml of 0.1 M Tris, pH 7.5, with or without β -mercaptoethanol at a concentration of $10^{-3} M$. The lower curve (○—○) was obtained with an incubation mixture prepared as above (with β -mercaptoethanol) plus $100 \mu\text{g}$ of the disulfide interchange enzyme. Aliquots of 0.1 ml were removed from the incubation mixtures for assay of chymotrypsin activity.

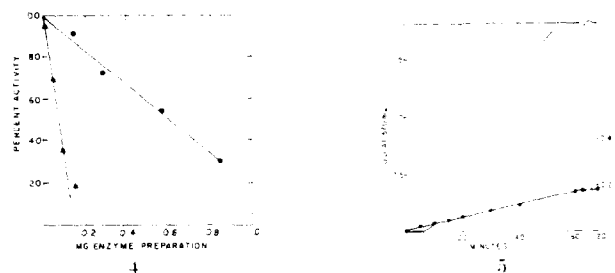


FIG. 4.—Inactivation of chymotrypsin by the disulfide interchange enzyme at two stages in its purification. Conditions of incubation were as described in the legend for Fig. 3 (with $100 \mu\text{g}$ of disulfide interchange enzyme and $10^{-3} M$ β -mercaptoethanol). The preparations of disulfide interchange enzyme were obtained after filtration on Sephadex G-200 (●—●) and after chromatography on DEAE-Sephadex (○—○).

FIG. 5.—Treatment of insulin with the disulfide interchange enzyme. The incubation mixture contained 4 mg of insulin per ml and $80 \mu\text{g}$ of the disulfide interchange enzyme per ml in 0.1 M Tris, pH 7.2, $10^{-3} M$ β -mercaptoethanol at room temperature. The turbidity of this solution was followed with a Cary model 15 spectrophotometer, using, in the reference cuvette, the same incubation mixture but without enzyme (upper curve, left-hand scale). The content of sulfhydryl groups in 1-ml aliquots of the incubation mixture after removal of β -mercaptoethanol is shown in the lower curve (right-hand scale).

zyme, as demonstrated by loss in the ability of the latter enzyme to catalyze the reactivation of reduced RNase. Koshland and Mozersky have shown²⁷ that chymotrypsin is highly resistant to spontaneous disulfide interchange, with only slow interchange occurring in 7.5 *M* urea. The action of the disulfide interchange enzyme on chymotrypsin was therefore studied after the latter enzyme had been exposed to 8 *M* urea. Following Martin and Frazier,²⁸ chymotrypsin (1 mg/ml) was incubated in 8 *M* urea, containing 0.2 *M* CaCl₂ and 0.1 *M* acetate buffer, pH 4.0, for 20 min. Under these conditions the inactivation is reversible and, in our hands, dilution with 100 vol of 0.1 *M* Tris buffer, pH 7.5, with or without 10⁻³ *M* β-mercaptoethanol, rapidly restored 85 per cent of the original chymotrypsin activity. When the diluting solution contained the disulfide interchange enzyme and 10⁻³ *M* β-mercaptoethanol, rapid inactivation of chymotrypsin was observed. As is shown in Figure 3, the inactivation was dependent on the presence of β-mercaptoethanol and rather large quantities of the microsomal enzyme. The fact that inactivation of chymotrypsin occurred mainly during the first minute suggests that the urea-treated protease remains in a denatured and inactive form for only a very short period after dilution. The inactivation by the enzyme at two stages in its purification is shown in Figure 4. A similar difference in specific activities was found when the two preparations were tested for their ability to catalyze the reactivation of reduced RNase.

If the inactivation of chymotrypsin is due to disulfide interchange and the production of random interchain and intrachain sets of disulfide bonds, a necessary control experiment is to test the effect of the enzyme on chymotrypsinogen, which presumably contains, in its single polypeptide chain, the information for the formation of the "correct" disulfide bonds. Chymotrypsinogen A was incubated in 8 *M* urea, as in the case of chymotrypsin, diluted 100-fold into Tris buffer containing 10⁻³ *M* β-mercaptoethanol and disulfide interchange enzyme (final concentrations: 1 mg enzyme and 10 μg chymotrypsinogen/ml), and incubated for 20 min at room temperature. Trypsin (0.5 μg/ml) was then added, and the appearance of chymotrypsin activity was followed by periodic assays. The theoretical level of chymotrypsin activity was achieved after 7 hr at 24°, demonstrating that chymotrypsinogen is not susceptible to inactivation by the disulfide interchange enzyme.

Insulin: The aggregating effect of the disulfide interchange enzyme leads to precipitation of insulin (enzyme:insulin, w/w, 1:50; 10⁻³ *M* β-mercaptoethanol, 0.1 *M* Tris buffer, pH 7.2), as illustrated in Figure 5. The physical properties of the precipitate changed with time, becoming progressively less soluble in 8 *M* urea or 1 per cent sodium dodecylsulfate. Samples of 1 ml, containing 4 mg of insulin, taken at the times indicated in Figure 5, were precipitated by the addition of 10 vol of acid acetone (1 *N* HCl:acetone, 1:39), washed twice with acid acetone, and dissolved in 0.1 *M* Tris buffer, pH 7.8, containing 1 per cent sodium dodecylsulfate and DTNB (10⁻³ *M*). As shown in Figure 5, free sulfhydryl groups appeared slowly during the incubation, reaching a final level of approximately 0.3 free SH group/mole of insulin. The precipitate formed at the end of 1 hr of incubation was washed three times with water, oxidized with performic acid, and subjected to hydrolysis by trypsin (1:100 w/w) in 0.1 *M* NH₄HCO₃ at 37° for 8 hr. Such treatment would be expected to have no effect on the oxidized A chain of insulin but to yield free alanine and two peptide fragments from the oxidized B chain. The results

TABLE 1
AMINO ACID ANALYSES* OF PRECIPITATES FORMED BY ACTION OF DISULFIDE INTERCHANGE
ENZYME ON INSULIN

Amino acid	50-Min precipitate	2-Hr precipitate	Insulin observed	A chain (theory)	B chain (theory)
Lys†	(1)	(1)	(1)	0	1
His	1.73	1.95	1.99	0	2
Arg	1.04	1.19	1.07	0	1
CysO ₃	3.65	5.57	—	—	—
Asp	1.91	2.13	3.25	2	1
Thr	1.10	1.10	0.95	0	1
Ser	1.77	1.69	2.67	2	1
Glu	4.82	5.64	7.61	4	3
Pro	1.29	1.24	1.12	0	1
Gly	3.75	4.23	4.24	1	3
Ala	2.68	2.89	3.12	1	2
1/2 Cys	—	—	5.48	4	2
Val	3.76	4.44	4.52	2	3
Met	0	0	0	0	0
Ilu	0.26	0.34	0.50	1	0
Leu	5.00	6.11	6.06	2	4
Tyr	1.90	2.85	3.90	2	2
Phe	3.18	3.49	2.91	0	3

* Uncorrected for destruction during hydrolysis.

† The relative amounts of the amino acids were calculated on the basis of lysine as 1.00.

of high-voltage electrophoresis of the digests of the oxidized precipitate showed the components expected from both chains. A second portion of the oxidized precipitate was hydrolyzed for amino acid analyses. The results, shown in Table 1, indicate that the precipitate formed in the presence of the enzyme contains both A and B chains of insulin. However, some enrichment for those amino acids present only in the B chain is evident, particularly in the early (50-min) precipitate. This finding suggests that during the process of interchange new intrachain disulfide bonds are formed, leading to separation of the A and B chains. This conclusion is consistent with the greater ease of reduction of interchain disulfide bonds in insulin²⁹ and the lower solubility of the B chain at pH 7.2.³⁰

A qualitative estimation of the rate and extent of disulfide interchange catalyzed by the enzyme was obtained by the preparation of peptide maps of peptic digests of insulin and of precipitates and material remaining in solution after 50 min and after 2 hr of incubation (6 mg insulin and 0.06 mg enzyme/ml, 0.1 M Tris, pH 7.2). The soluble fractions were precipitated and washed with acid acetone as were the precipitates. After removal of the acetone-HCl *in vacuo*, 10-mg samples of each fraction and of untreated insulin were dissolved or suspended in 5 per cent formic acid and digested with pepsin. The digests were diluted tenfold with water, lyophilized, and 2.5-mg aliquots were applied to Whatman 3 MM paper for peptide mapping. Duplicate maps of each fraction were stained as described above.

The peptide maps were identical except for those components staining positively for disulfide bonds. The disulfide-positive components characteristic of insulin were still faintly visible in the soluble and insoluble fractions after 50 min of incubation although a number of new peptides containing disulfide bonds, having different mobilities, were already present in large amounts. After 2 hr of incubation, digests of both the soluble and insoluble fractions showed a considerable fraction of the disulfide-positive material remaining at the origin, while the rest was present in new locations on the map.

Evidence for change in the three-dimensional structure of insulin after treatment

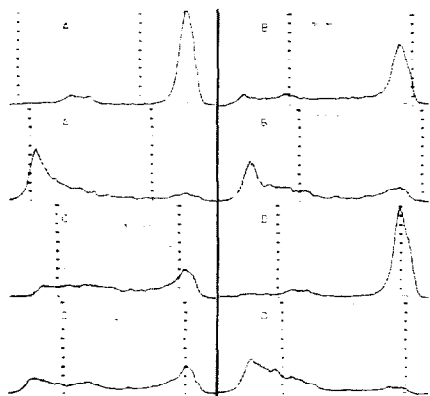


FIG. 6.—Immunassay of insulin treated with the disulfide interchange enzyme. Curve *A*: insulin alone. Curve *B*: insulin and β -mercaptoethanol. Curve *C*: insulin, β -mercaptoethanol, and disulfide interchange enzyme. Curve *D*: insulin and disulfide interchange enzyme. Curves *A'*, *B'*, *C'*, and *D'* are the same as the corresponding curves except that antiserum was added after 30 min of incubation at 37°. The quantities of the various components are given in the text.

in the presence of antibodies, the antibody-bound insulin moves with the front (Fig. 6, *A, A'*). The addition of enzyme alone has no effect (Fig. 6, *D, D'*) and only a small effect results with β -mercaptoethanol alone (Fig. 6, *B, B'*). In the presence of both enzyme and β -mercaptoethanol the chromatographic pattern of insulin is markedly changed (Fig. 6, *C*). However, this pattern is not affected by the presence of antibodies (Fig. 6, *C'*) and almost no antibody-bound insulin is observed. These observations, together with those described above, indicate that extensive disulfide interchange has taken place, with disruption of the antigenically specific structure of the insulin molecule.

Discussion.—The information now available indicates that the enzyme used in these studies catalyzes a process of sulfhydryl-disulfide interchange. For example, an RNase derivative, prepared by oxidation in urea which causes "incorrect" pairing of the eight sulfhydryl groups of the reduced polypeptide chain, is rapidly converted to the native protein by the enzyme. The process requires the presence of small amounts of a thiol reagent such as β -mercaptoethanol or, alternatively, the introduction into the randomly disulfide-bonded derivative of a few sulfhydryl groups prior to addition of the enzyme.

It is known that the disulfide interchange reaction is catalyzed by small amounts of thiol compounds.³¹ Reactivation of the fully oxidized inactive RNase derivative occurs in the presence of β -mercaptoethanol alone, although at a much slower rate than in the presence of enzyme. The disulfide interchange is accompanied by a rapid rearrangement of tertiary structure, the sole driving force for which appears to be the highly favorable free energy of conformation of the native structure as compared with those of other three-dimensional arrangements.

If both the tertiary structure and the pairing of half-cystine residues in a protein

with the disulfide interchange enzyme was obtained by measurements of antigenicity. A solution of I¹³¹-labeled insulin (1 μ g/ml) containing bovine serum albumin (50 mg/ml) was incubated with and without enzyme (50 μ g/ml), and in the presence and absence of 10^{-3} *M* β -mercaptoethanol. Incubations were carried out in 0.1 *M* Tris buffer, pH 7.2, at 37° for 30 min. At the end of this period, 0.05 ml of the diluted antiserum was added to half of the incubation mixture. After 48 hr at 4°, the material that had been treated with antiserum and the control fraction were chromatographed, and the distribution of radioactivity was scanned on each chromatogram (Fig. 6).

Under the conditions of chromatography the small amount of insulin used remains at the origin, while, in

are a predetermined consequence of the amino acid sequence, cleavage of one or more peptide bonds might be expected to upset the delicately balanced set of interactions required to achieve the native structure. As we have observed in these studies, native RNase and chymotrypsinogen are not altered by the disulfide interchange enzyme. On the other hand, the three-chained structures of the chemically produced "C-protein" derivative of RNase, and the product of enzymic activation of chymotrypsinogen, chymotrypsin, are rapidly modified by the enzyme. These results suggest that information present in the original single-chained precursors is missing after fragmentation of the chains. Thus the disulfide interchange enzyme appears to constitute a useful "thermodynamic probe" for testing the intrinsic stability of disulfide-bonded polypeptides in general.

Our results with insulin support the view that the hormone is originally synthesized as a single-chained protein and later converted to the two-chained form by a zymogen-like conversion. Such a mechanism would be consistent with the recent observations of Markus on the conformational changes induced in insulin by nondenaturing electrolytic reduction,³² and with reports of low yields of insulin following oxidation of mixtures of reduced A and B chains.³³⁻³⁶

We wish to thank Dr. Frank Tietze for the samples of I¹³¹-labeled insulin and antisera and for his helpful advice concerning the immunoassay of insulin. We are also grateful to Mrs. Diana Trundle for her technical assistance.

* Visiting scientist, on leave from Weizmann Institute of Science, Rehovoth, Israel.

† International fellow, USPHS; on leave from University of Naples, Italy.

¹ Kendrew, J. C., *Science*, **139**, 1259 (1963).

² Perutz, M. F., *Science*, **140**, 863 (1963).

³ Anfinsen, C. B., in *Enzyme Models and Enzyme Structure*, Brookhaven Symposia in Biology, No. 15 (1962), p. 194.

⁴ Epstein, C. J., R. F. Goldberger, and C. B. Anfinsen, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 439.

⁵ Fraenkel-Conrat, H., and B. Singer, *Biochim. Biophys. Acta*, **33**, 359 (1959).

⁶ Anderer, F. A., *Z. Naturforsch.*, **14b**, 642 (1959).

⁷ Harrison, S. C., and E. R. Blout, *J. Biol. Chem.*, in press.

⁸ White, F. H., Jr., *J. Biol. Chem.*, **236**, 1353 (1961).

⁹ Imai, K., T. Takagi, and T. Isemura, *J. Biochem.*, **53**, 1 (1963).

¹⁰ Goldberger, R. F., and C. J. Epstein, *J. Biol. Chem.*, **238**, 2988 (1963).

¹¹ Isemura, R., T. Takagi, V. Maeda, and K. Yutani, *Biochem. J.*, **53**, 155 (1963).

¹² Frattali, V., R. F. Steiner, D. B. S. Millar, and H. Edelhoch, *Science*, **199**, 1186 (1963).

¹³ Goldberger, R. F., C. J. Epstein, and C. B. Anfinsen, *J. Biol. Chem.*, **239**, 1406 (1964); Vene-
tianer, P., and F. B. Straub, *Biochim. Biophys. Acta*, **67**, 166 (1963).

¹⁴ Givol, D., R. F. Goldberger, and C. B. Anfinsen, *J. Biol. Chem.*, **239**, 3114 (1964).

¹⁵ Anfinsen, C. B., and E. Haber, *J. Biol. Chem.*, **236**, 1361 (1961).

¹⁶ Haber, E., and C. B. Anfinsen, *J. Biol. Chem.*, **237**, 1839 (1962).

¹⁷ Gross, E., and B. Witkop, *J. Biol. Chem.*, **237**, 1856 (1962).

¹⁸ Anfinsen, C. B., R. R. Redfield, W. L. Choate, J. Page, and W. R. Carroll, *J. Biol. Chem.*, **207**,
201 (1954).

¹⁹ Hummel, B. C. W., *Can. J. Biochem. Physiol.*, **37**, 1393 (1959).

²⁰ Potts, J. T., Jr., D. M. Young, C. B. Anfinsen, and A. Sandoval, *J. Biol. Chem.*, **239**, 3781
(1964).

²¹ Berson, S. A., R. S. Yalow, A. Bauman, M. A. Rotschild, and K. Newerly, *J. Clin. Invest.*, **35**,
170 (1956).

²² Toennies, G., and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).

²³ Hirs, C. H. W., *J. Biol. Chem.*, **219**, 611 (1956).

- ²⁴ Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- ²⁵ Ellman, G. L., *Arch. Biochem. Biophys.*, **82**, 70 (1959).
- ²⁶ Epstein, C. J., R. F. Goldberger, M. D. Young, and C. B. Anfinsen, *Arch. Biochem. Biophys.*, Suppl. 1, 223 (1963).
- ²⁷ Koshland, D. E., and S. M. Moersky, *Federation Proc.*, **23**, 609 (1964).
- ²⁸ Martin, C. J., and A. R. Frazier, *J. Biol. Chem.*, **238**, 3268 (1963).
- ²⁹ Markus, G., in *Protides of the Biological Fluids II*, ed. M. Peters (1963).
- ³⁰ Tomizawa, H. H., *Federation Proc.*, **20**, 190 (1961).
- ³¹ Ryle, A. P., and F. Sanger, *Biochem. J.*, **60**, 535 (1954).
- ³² Markus, G., *J. Biol. Chem.*, **239**, 4163 (1964).
- ³³ Dixon, G. A., and A. C. Wardlaw, *Nature*, **188**, 721 (1960).
- ³⁴ Meienhofer, J., E. Schuabel, H. Brenner, O. Brinkhoff, R. Zabel, W. Snoka, H. Keostermeyer, D. Brendenberg, T. Akuda, and H. Zahn, *Z. Naturforsch.*, **18b**, 1120 (1963).
- ³⁵ Niu, C., Y. Kung, W. Huang, L. Ke, C. Chen, Y. Chen, Y. Du, R. Jiang, C. Tsou, S. Hu, S. Chu, and K. Wang, *Sci. Sinica*, **13**, 1343 (1964).
- ³⁶ Tietze, F., personal communication.